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Identification and characterization of nuclear receptors from the red flour beetle, *Tribolium castaneum*

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Abstract

Nuclear receptors (NRs) are classified by the presence of a well-conserved DNA-binding domain and a less conserved ligand-binding domain and function as key control points in diverse signaling and metabolic pathways. NRs are switched on and off by small molecule ligands with properties similar to insecticides. Therefore, NRs are attractive targets for developing new insecticides. Nineteen canonical and two Knirps family NRs were identified in the genome of *Tribolium castaneum*. RNAi analysis showed that 10 out of the 19 canonical NRs, TcE75, TcHR3, TcHR4, TcEcR, TcUSP, TcFTZ-F1, TcHR51, SVP, TcHR38, TcHR39 are important for metamorphosis. Knocking down the expression of five NRs, TcTll, TcDsf, TcHNF4 and TcHR78 caused defects in production of offspring. TcHNF4, TcHR78, TCHR51 and TcDsf affected egg production and TcTll affected embryonic development. Knocking down the expression of non-canonical NR Knirps-like affected adults and caused reduction in egg production. The other Knirps family member, Eagle, and five canonical NRs, TcE78, TcHR83, TcHR96, TcPNR-like and TcERR did not show much effect on metamorphosis or production of offspring. Quantitative real-time reverse transcriptase analysis showed that the mRNA levels of all NRs tested were reduced in DsRNA injected larvae when compared to their levels in control larvae injected with bacterial *malE* DsRNA suggesting that the RNAi worked well but reduction in expression levels of some of the NRs did not affect metamorphosis or production of offspring.

Keywords: Metamorphosis; Ligand; Ecdysone; Transcription; Molting

1. Introduction

Nuclear receptors (NRs) are a group of proteins characterized by the presence of two functional domains, a highly conserved DNA-binding domain (DBD) consisting of two C4 type zinc fingers and a less conserved ligand-binding domain (LBD) containing a ligand-binding pocket, dimerization domain and activation domain. The 48 members of the NR superfamily in humans include receptors for steroid hormones, thyroid hormones, vitamin D and retinoic acid. About half of human NRs function through known natural ligands and the other half, so-called orphan receptors have no known natural ligands (Benoit et al., 2006; Flamant et al., 2006; Germain et al., 2006; Lu et al., 2006). NRs play key roles in embryonic and post-embryonic development and reproduction functioning as key control points in diverse signaling and metabolic pathways such as metabolism (DeLuca, 2004), sex determination (Iyer and McCabe, 2004), circadian rhythm, aging (Pardee et al., 2004) and xenobiotic detoxification (Willson and Kliewer, 2002). NRs are switched on and off by small molecule ligands with the properties similar to drugs. Therefore, NRs are attractive targets for the prevention and treatment of diverse diseases such as cancer, coronary heart disease, and diabetes. In fact, several known anti-cancer drugs, such as tamoxifen or flutamide, target NRs, and many more are expected to reach the market in the near future.

Abbreviations: 20E, 20-hydroxyecdysone; Coup-TF1, chicken ovalbumin upstream promoter transcription factor 1; DHR, *Drosophila* hormone receptor; EcR, ecdysone receptor; ERRb, estrogen-related receptor beta; HNF4, hepatocyte nuclear factor 4; LRH1, liver receptor homolog 1; NURR1, NR4A2 receptor; PNR, photoreceptor-specific nuclear receptor; Rev-ERBA, nuclear receptor-related protein coded on the opposite strand of the thyroid hormone receptor gene; RORB, retinoid-related orphan receptor beta; SF-1, steroidogenic factor 1; Tll, tailless homolog; TR2, NR2C1 receptor; USP, ultraspiracle; VDR, vitamin D receptor

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The number of NRs identified in sequenced genomes varies considerably. Two hundred and eighty-four NRs have been identified in nematode, *Caenorhabditis elegans* compared to 18 identified in the genome of fruit fly, *Drosophila melanogaster* (Maglich et al., 2001). In addition to 18 canonical NRs, *D. melanogaster* genome also contains three additional NRs (the Knirps family) that contain well-conserved DBD but lack the LBD (Adams et al., 2000). Recently sequenced *Anopheles gambiae* and *Apis mellifera* genomes contain all 18 canonical NRs identified in *D. melanogaster* genome (Holt et al., 2002; Velarde et al., 2006). An additional PNR-like receptor not present in *D. melanogaster* has been identified in *A. mellifera* genome (Velarde et al., 2006). Majority of functional studies on NRs in *D. melanogaster* have been focused on embryogenesis and metamorphosis. Several members of this family are regulated by 20E and play critical roles in molting and metamorphosis. Most NRs are also expressed in adults, except for a few studies, very little is known about the function of NRs in adult insects (Sullivan and Thummel, 2003). The mRNAs for several NRs including seven-up (SVP), ultraspiracle (USP), HR3, FTZ-F1 and HNF4 were detected in the brains of adult honeybee (Velarde et al., 2006). In the yellow fever mosquito, *Aedes aegypti*, EcR, USP, SVP and E75 play critical roles in 20-hydroxyecdysone signal transduction for regulation of egg production after blood meal (Raikhel et al., 2002).

Red flour beetle *Tribolium castaneum* could be a good model insect to study the function of NRs in adult insects because (1) they are easy to culture, (2) they have a short life cycle, (3) females have high fecundity, (4) they are amenable for genetic crosses, (5) genome is sequenced and (6) injection of double-stranded RNA (DsRNA) during larval stages results in silencing of genes during adult stages (Tomoyasu and Denell, 2004). We therefore, identified NRs in the genome sequence of *T. castaneum* and studied their function using RNA interference (RNAi).

2. Materials and methods

2.1. Rearing and staging of beetles

Strain GA-1 of *T. castaneum* (Haliscak and Beeman, 1983) was reared on organic wheat flour containing 10% yeast at 32 °C under standard conditions (Beeman and Stuat, 1990). Newly ecdysed final instar larvae were separated based on the white head character soon after molting and staged from that time onwards.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from five to six larvae for each sample using the TRI reagent (Molecular Research Center Inc., Cincinnati, OH). The RNA was treated with DNase I (Ambion Inc., Austin, TX) and cDNA synthesis was performed using 2 μg of total RNA and iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a 20 μl reaction volume.

2.3. Double-stranded RNA synthesis

Genomic DNA was used as a template to amplify regions of NRs and the PCR product was used for DsRNA synthesis. Genomic DNA was extracted from *T. castaneum* adults and purified using the DNeasy Tissue Kit (QIAGEN). Primers containing NR-specific sequence and the T7 RNA polymerase promoter at their 5′ ends (Table 1) designed using Primer3 (http://frodo.wi.mit.edu/) were used to PCR amplify 200–600 bp regions of NRs. In order to PCR amplify these fragments using genomic DNA as the PCR template, the region of the NR targeted by the dsRNA was selected from within one exon. The PCR products were then used as template to synthesize DsRNA. MEGAscript RNAi Kit (Ambion Inc., Austin, TX) was used for DsRNA synthesis. For annealing DsRNA, the reaction products were incubated at 75 °C for 5 min and cooled to room temperature over a period of 60 min. After the treatment with RNase and DNase, dsRNA was purified by phenol/chloroform extraction followed by ethanol precipitation.

2.4. Microinjection

One-day-old final instar larvae were anesthetized with ether vapor for 4–5 min and lined on a glass slide covered with double-sticky tape. The DsRNA was injected into the dorsal side of the first or second abdominal segment using an injection needle pulled out from a glass capillary tube using needle puller (Idaho technology). 0.8–1 μg (0.1 μl) dsRNA was injected into each larva. The DsRNA prepared using 800 bp bacterial *malE* gene as a template was used as a control. Injected larvae were removed from the slide and raised in whole wheat flour at 32 °C.

2.5. Quantitative real-time PCR (qRT-PCR)

Relative mRNA levels of selected NRs after injection of DsRNA were determined by qRT-PCR. cDNA prepared using RNA isolated from control *malE* DsRNA or NR DsRNA injected larvae, primers designed based on NR sequences (Table 2) and MyiQ single color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) were used to perform qRT-PCR. The primers for qRT-PCR were designed based on the sequence from the NR regions that are outside the DsRNA target regions. qRT-PCR reactions were performed using a common program as follows: initial incubation of 95 °C for 3 min was followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 30 s. Standard curves were obtained using a 10-fold serial dilution of pooled cDNA. Quantitative mRNA measurements were performed in triplicate and normalized to an internal control of *T. castaneum* ribosomal protein *rp49* mRNA.

Table 1
Primers used for dsRNA synthesis

Gene	Length of dsRNA (bp)	Forward primer	Reverse primer
TcE75	473	taatacgactcactatagggGGACTGTTTTGTTCGGTCGT	taatacgactcactatagggGAGGAGCGTTGGTTACGTTC
TcE78	203	taatacgactcactatagggAATCCGACTCCGAAACGAAG	taatacgactcactatagggCAGCCAACACCTTTCCTGTT
TcHR3	220	taatacgactcactatagggATCAGACGCACACACACGTA	taatacgactcactatagggGATCTGATCGTCTTG GGACA
TcEcR	304	taatacgactcactatagggGCCTCCGGTTACCACTACAA	taatacgactcactatagggCTGGTTCAACCTTGATGACG
TcHR96	494	taatacgactcactatagggCTTCAATGACCAGTGCGAGA	taatacgactcactatagggCGACGAAGTTGGGTCGTTAT
TcHNF4	247	taatacgactcactatagggGGCCAACTCCTGTTAACGAT	taatacgactcactatagggGCAGTTGTTTCCCAAAAGGA
TcRXR	229	taatacgactcactatagggGAGGGATAAAGTCGGTGCAA	taatacgactcactatagggGCCCTCCAACATCTCCATTA
TcHR78	419	taatacgactcactatagggAGCCAGGCAAGTATCGAAGA	taatacgactcactatagggGCTAGCCCCAAAGTGAACAG
TcTailless	466	taatacgactcactatagggTTGGAGGAAAGCTGGCTAGA	taatacgactcactatagggGCGTGTCCCTGATCACTTTT
tcDHR51	215	taatacgactcactatagggCCCATTACATGCCAGAATCC	taatacgactcactatagggTGAGAACATCACCAGGAAGC
TcDissatisfaction	439	taatacgactcactatagggAACTGCAAGCATCCTCCAAC	taatacgactcactatagggAGCTGCAACATCTCCCAAGT
TcHR83	431	taatacgactcactatagggCCAACCCAATCCAGGAACTA	taatacgactcactatagggATGCAACACTACAGCCGATG
TcPNR-like	354	taatacgactcactatagggACCACCTCATCCAGCAATGT	taatacgactcactatagggGCTGTCAGGACGAACAGCTC
TcSeven up	199	taatacgactcactatagggCATTCCCACCAGCTTGAACT	taatacgactcactatagggACAACGCACTCGATGTTCTG
TcERR	231	taatacgactcactatagggTCCAAACACCGGACTCTTTC	taatacgactcactatagggCTCATCGAGGGTGAAGTCGT
TcHR38	581	taatacgactcactatagggCTGCAAGGGCTTCTTCAAAC	taatacgactcactatagggTTTGACACTGCTGCTTGTCC
TcFTZ-F1	384	taatacgactcactatagggCAGGTCAACACCTCCAACCT	taatacgactcactatagggGCCCAATCGACTTGAGAGAA
TcDHR39	357	taatacgactcactatagggGCCGCCATATGCACAGTATT	taatacgactcactatagggACGCTGGACACTTCTTCCTT
TcHR4	373	taatacgactcactatagggCCGCTCAACATGGAGAGACT	taatacgactcactatagggCCATTCCCTGCTCGATACAT
TcKnirps-like	436	taatacgactcactatagggGTTCTTCGGGAGGTCGTACA	taatacgactcactatagggAGAGTCGGAGTTGTGCGATT
TcEagle	394	taatacgactcactatagggAGTGCCTTTTGGTCGGTATG	taatacgactcactatagggACGGAAGGAGCGTTGACTAA

Table 2
Primers used in qRT-PCR

Gene	Forward primer	Reverse primer
TcE78	GTGATGATGTTCGACGATGG	TGAACCTTCAGAGCCTCGAT
TcHR96	GCACGAGCGTTTCATTAACA	TCATTGMCCTGTGCAGCTC
TcHNF4	CAAGGCCCTACGTTACCAM	TTGACTGAAGAGCGGGAAGT
TcHR78	GGCTTATCGTGGGGGTTTAT	ATACCTCCCTGCGCCTTTAT
TcDsf	CTACCGGCCGATATTCACAC	GGGACAAGAAGAAGCAGTCG
TCHR83	ACAAATGCCTCAGTCCGAAG	GGGATTCCTCCGTTCGTATT
TcPNR-like	CCGAACATGCGCGTCTTMA	TGAACCGCTGGTAGGGTMG
TcERR	ATCAAACGTGTTGCCATGTG	CCGACAAMTCGAGTCCCTA
Eagle	CATCGACCTCTCGCTCAAG	AAAGGCACAGGTTTGAGTGG
rp49	TGACCGTTATGGCAMCTCA	TAGCATGTGCTTCGTTTTGG

2.6. NR effects on reproduction and/or embryogenesis

Healthy females developed from DsRNA injected larvae were mated with males developed from uninjected larvae. Adults were removed from the flour at 2 weeks after initiation of matting. The number of eggs was counted. After incubation of eggs for 1 additional week, the number of larvae developed from eggs laid by each mated pair was counted.

3. Results

3.1. Identification of NRs in T. castaneum

We searched *T. castaneum* genome sequence deposited in Beetlebase (www.bioinformatics.ksu.edu/BeetleBase/), NCBI (www.ncbi.nlm.nih.gov/projects/genome/seq/BlastGen/BlastGen.cgi?taxid=7070), and human genome sequence center at Baylor College of Medicine (http://www.hgsc.bcm.tmc.edu/blast.hgsc?organism=13) and identified 19 canonical NRs and two Knirps family members (Table 3). The NR superfamily members present in the databases were identified by performing BLAST search with 66 amino acid DBD motif. Individual NRs were identified by performing BLAST search with each potential *T. castaneum* NR hit. The consensus gene set predicted from the *T. castaneum* genome assembly and deposited at human genome sequence center at Baylor College of Medicine (http://www.hgsc.bcm.tmc.edu/blast.hgsc?organism=13) contained all the 21 NRs identified (Table 3). The gene sets deposited in the GenBank contains 18 NRs and the gene sets deposited in the beetlebase contains 19 NRs. The identification numbers for NRs in all three databases are shown in Table 3.

Table 3
Nuclear receptors identified in *T. castaenum*

Nomenclature	*Tribolium*	*Drosophila*	Human	Tc Vs.Dm DBD/LBD (%)[a]	Tc Vs.Hs DBD/LBD (%)[b]	GLEAN#[c]	GenBank accession no.	Beetlebase name
NR0A2	Knirps-like	Knirps-like	NP	95/–	NP	TC_03413	XP_968510	TG1703
NR0A3	Eagle	Eagle	NP	89/–	NP	TC_03409	XP_968345	TG8505
NR1D3	E75	E75	REV-ERBA	100/54	83/42	TC_12440	XP_971362	TG5362
NR1E1	E78	E78	REV-ERBA	89/14	78/35	TC_03935	XP_966635	TG8961
NR1F4	HR3	DHR3	RORB	94/62	73/34	TC_08909	XP_974561	TG1094
NR1H1	EcR	EcR	FXR, LXR	88/67	85/39, 68/40	TC_12112, TC_12113	XP_969064	TG3244
NR1J1	HR96	DHR96	VDR	82/67	59/27	TC_10645	XP_968487	TG7971
NR2A4	HNF4	HNF4	HNF4A	94/76	89/66	TC_08726	XP_968613	TG2304
NR2B4	USP	USP	RXRA	92/44	86/62	TC_14027, TC_14028	XP_972475	TG3456
NR2D1	HR78	DHR78	TR2	92/32	80/45	TC_04598	NA	TG4611
NR2E2	Tailless	Tailless	TLX	79/39	77/40	TC_00441	NP_001034502	TG3748
NR2E3	HR51	DHR51	TLX	97/50	85/43	TC_09378	XP_970391	TG3825
NR2E4	Dsf	Dsf	PNR	94/77	79/43	TC_01069	XP_975255	NA
NR2E5	HR83	DHR83	PNR	72/23	65/22	TC_10460	XP_973016	TG7403
NR2E6	PNR-like	NP	PNR	89/64(Am)	65/49	TC_13148		TG2512
NR2F3	Seven-up	Seven-up	COUP-TF1	94/95	92/93	TC_01722	XP_967537	TG5035
NR3B4	ERR	ERR	ERRb	88/49	80/47	TC_09140, TC_09141	NA	TG7271
NR4A4	HR38	DHR38	NURR1	97/79	91/63	TC_13146	NA	TG2508
NR5A3	FTZ-F1	FTZ-F1	LRH-1, SF-1	100/68	86/38, 86/32	TC_02550	XP_970369	TG7855
NR5B1	HR39	DHR39	LRH-1, SF-1	89/79	59/32, 59/30	TC_14986	XP_970387	TG7175
NR6A2	HR4	DHR4	GCNF	99/62	63/28	TC_00543	XP_974320	NA

NP, not present; NA, not available.

[a]Percent amino acid identity in the DNA-binding (DBD) and ligand-binding (LBD) region between in homologous proteins in *T. castaenum* and *D. melanogaster*.

[b]Percent amino acid identity in DBD and LBD between in homologous proteins in *T. castaenum* and *Homo sapiens*.

[c]Shows GLEAN number assigned to these genes in the consensus gene set predicted from the genome sequence, GLEAN was replaced with TC.

Overall, the NRs are well conserved in the four insect genomes sequenced so far with a few minor differences. Eighteen of the NRs identified in *T. castaneum* have homologues in *D. melanogaster*, *A. mellifera*, *An. gambiae* genomes. One additional PNR-like NR has been identified in *A. mellifera* and *T. castaneum* genomes but not in *D. melanogaster* or *An. gambiae* genomes. Both *D. melanogaster* and *A. mellifera* genomes contain three Knirps family members. In *T. castaneum*, only two members of Knirps family could be identified. The amino acid identity between *T. castaneum* and *D. melanogaster* as well as between *T. castaneum* and human NRs in the DBD and LBD are shown in Table 3.

In addition to 19 canonical NRs, two members of Knirps family NRs (TcKnirps-like and TcEagle) were identified in the *T. castaneum* genome. The Knirps identified in *T. castaneum* showed higher amino acid similarity with *D. melanogaster* Knirps-like when compared to its amino acid identity with *D. melanogaster* Knirps protein.

3.2. Analysis of function of NRs in T. castaneum

To determine the function of NRs in *T. castaneum*, we prepared DsRNA for 19 canonical NRs and the DsRNAs were injected into the last instar larvae. As shown in Table 4, 100% of the larvae injected with DsRNA for TcE75, TcHR3, TcEcR, TcUSP, TcFTZ-F1 and TcHR4 died prior to pupation. In addition, 60% of larvae injected with DsRNA for TcHR51 died prior to pupation. DsRNA prepared using 808 bp region of bacterial *malE* gene was used as a control and only 3 out of 58 larvae injected with this DsRNA died. These data suggest that TcE75, TcHR3, TcEcR, TcUSP, TcFTZ-F1, TcHR4 and TcHR51 play critical roles in larval–pupal metamorphosis. Some of the larvae injected with TcSVP, TcHR38 and TcHR39 died during larval–pupal metamorphosis and more of them died during pupal–adult metamorphosis. None of the larvae injected with TcSVP DsRNA became adults. About 25% of larvae injected with TcHR38 and TcHR39 DsRNA became adults. These data suggest that these three NRs play key roles during both larval–pupal as well as pupal–adult metamorphosis, but they are not as critical as the seven NRs (TcE75, TcHR3, TcEcR, TCUSP, TcFTZ-F1, TcHR4 and TcHR51) for larval–pupal metamorphosis.

The DsRNA injected insects showed various phenotypes depending on the NR injected. The larvae injected with TcE75, TcHR3, TcEcR, TcUSP, TcFTZ-F1 and TcHR4 DNA died at various stages during final instar larval stage

Table 4
Effect of silencing of nuclear receptor expression on the survival of beetles

	Gene name	No. of larvae injected	No. of larvae dead	Larvae dead (%)	No. of pupae	No. of pupae dead	Pupae dead (%)	No. of adults
1	TcE75	30	30	100	0	0	0	0
2	TcE78	57	14	25	43	9	16	34
3	TcHR3	30	30	100	0	0	0	0
4	TcEcR	30	30	100	0	0	0	0
5	TcHR96	57	15	26	42	6	11	36
6	TcHNF4	60	18	30	42	7	12	35
7	TcRXR	30	30	100	0	0	0	0
8	TcHR78	57	23	40	34	0	0	34
9	TcTll	59	25	42	34	2	3	32
10	TcHR51	58	35	60	23	7	12	16
11	TcDsf	60	21	35	39	0	0	39
12	TcHR83	40	2	2.5	38	2	6	36
13	TcPNR	61	6	10	55	0	0	55
14	TcSVP	58	22	38	36	36	62	0
15	TcERR	57	20	35	37	2	4	35
16	TcHR38	56	22	39	34	20	36	14
17	TcFTZ-Fl	30	30	100	0	0	0	0
18	TcHR39	57	18	32	39	25	44	14
19	TcHR4	58	58	100	0	0	0	0
	TcKnirps-like	60	22	37	38	11	18	27
	TcEagle	56	15	27	41	2	4	39
	MalE	58	3	5	55	1	2	54

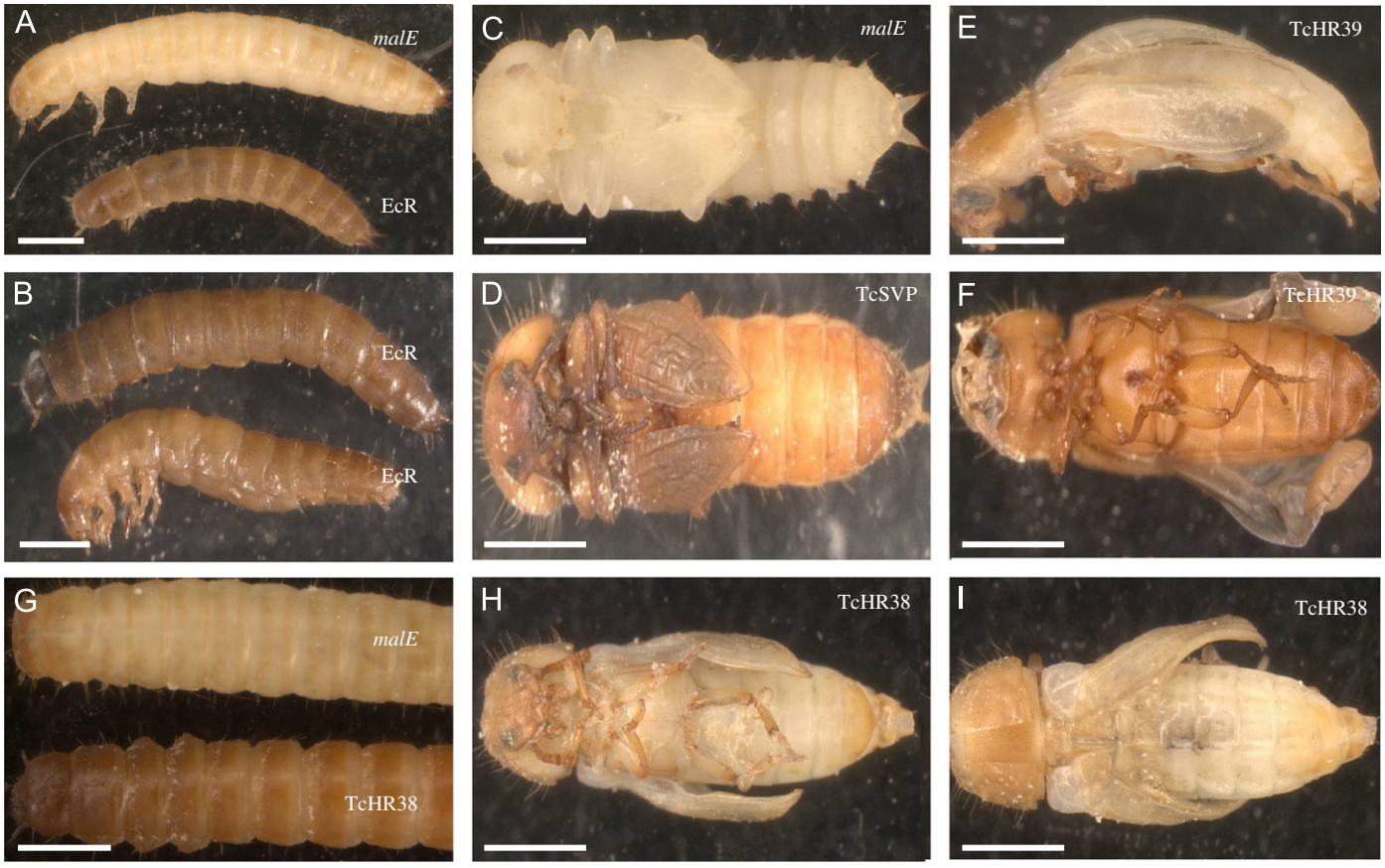

Fig. 1. Phenotypes observed after injection of NR DsRNA. Control *malE* or nuclear receptor DsRNA was injected into 1-day-old final instar larvae. Pictures shown are various abnormal phenotypes observed during larval and pupal stages. (A and B) The larvae died after EcR DsRNA injection; (C and D) abnormal phenotypes observed in pupae developed from seven-up (SVP) DsRNA injected larvae; (E and F) abnormal phenotypes observed in pupae developed from TcHR39 DsRNA injected larvae; (G) increased pigmentation observed in larvae injected with TcHR38 DsRNA; (H and I) abnormal pupae developed from TcHR38 DsRNA injected larvae. Scale bar: 1 mm.

and showed phenotypes similar to the images presented in Fig. 1A and B. Some of the EcR DsRNA injected larvae did not feed, remained smaller and died prior to entering quiescent stage (Fig. 1A). Some of the injected larvae entered quiescent stage and died during quiescent stage. Some of the larvae initiated molting process, slipped head capsule and got stuck at this stage and died (Fig. 1B). A few larvae injected with EcR DsRNA progressed half way through the molting process and died at this stage (Fig. 1B). The larvae injected with TcUSP DsRNA showed phenotypes similar to the phenotypes showed by TcEcR DsRNA injected larvae. Some of the TcUSP DsRNA injected larvae died prior to entering quiescent stage. Some of the larvae injected with TcUSP DsRNA entered quiescent stage and died during this stage. Some of the TcUSP DsRNA injected larvae initiated molting and died during molting. Majority of larvae injected with TcE75, TcHR3, TcHR4 or TcFTZ-F1 DsRNA died during quiescent stage. A few larvae injected with these DsRNA died during molting. Sixty percent of the larvae injected with TcHR51 DsRNA died during quiescent stage prior to pupation. Twelve percent of the pupae also died during pupal stage. Twenty-eight percent of the larvae injected with TcHR51 DsRNA became adults.

In contrast, majority of larvae injected with TcSVP pupated but the pupae did not develop further after larval–pupal ecdysis. Most of the pupae remained untanned or less tanned and the wing development did not progress normally as a result these pupae showed only small wings covering only thoracic region and died at this stage (Fig. 1D). About 68% of larvae injected with TcHR39 became pupae but majority of these pupae showed abnormal wing development and died at this stage (Fig. 1E and F). The larvae injected with TcHR38 DsRNA were darker but most of them became pupae (Fig. 1G). However, most of the pupae showed defects especially in development and sclerotization of wings (Fig. 1H and I). Most of these insects died during the pupal stage. More than 60% of the larvae injected with DsRNA for the other nine NRs (TcE78, TcHR96, TcHNF4, TcHR78, TcHR83, TcTll, TcDsf, TcPNR-like and TcERR) became healthy adults suggesting that these NRs are not critical for molting and metamorphosis (Table 4).

About 37% of the larvae injected with TcKnirps-like DsRNA and 27% of the larvae injected with Eagle DsRNA died during larval stage. Additional 11% of larvae injected with TcKnirps-like DsRNA died during the pupal stage. About 27% of TcKnirps-like DsRNA and 39% of TcEagle DsRNA injected insects emerged as adults. Both larvae and pupae died as a result of Knirps-like or Eagle knock-down did not show any defects associated with molting and metamorphosis. The DsRNA injected insects remained at these stages, desiccated and died. It is not clear whether Knirps-like or Eagle is interfering with molting or metamorphosis.

3.1. Effect of NRs on production of offspring

The number of larvae hatched from eggs laid by each pair over a 2-week period was determined after mating female adults developed from DsRNA injected larvae with males developed from larvae that were not injected. As shown in Fig. 2, females developed from the larvae injected with TcTll and TcHR51 DsRNA produced no offspring. The females developed from larvae injected with TcHNF4, TcHR78, TcDsf or TcKnirps-like produced fewer offspring when compared to the females developed from *malE* DsRNA injected larvae (Fig. 2). Females developed from TcHR83, TcHR96, TcE78, TcPNR-like, TcERR and TcEagle produced similar number of offspring when compared to females developed from *malE* DsRNA injected larvae (Fig. 2).

We then determined whether the reduction in number of offspring produced by females developed from insects injected with TcTll, TcHR51, TcHNF4, TcHR78, TcDsf or TcKnirps-like DsRNA is due to the effect of knock-down in the expression of these genes on the egg production or embryonic development. The females developed from larvae injected with TcHR51 did not lay any eggs and the females developed from the larvae injected with TcHNF4, TcHR78 or TcDsf laid fewer eggs (an average of 10 for three pairs compared to 30 laid by three control females developed from *malE* DsRNA injected larvae). The ovaries dissected from females developed from larvae injected with TcHR51, TcHNF4, TcHR78 or TcDsf showed similar morphology to those dissected from females developed from larvae injected with *malE* DsRNA. These data suggest that TcHR51, TcHNF4, TcHR78 and TcDsf play key roles in reproduction and egg development. The females developed from larvae injected with TcTll DsRNA laid similar number of eggs laid by the females developed from *malE* DsRNA injected larvae. But the larvae did not hatch from the eggs laid by the females developed from the larvae injected with TcTll DsRNA suggesting that Tll plays key role in embryonic development. Knirps-like RNAi affected adult development. RNAi animals which survived to adult stage died during the first week. The females developed from larvae injected with Knirps-like DsRNA and mated with healthy males laid very few eggs. These data suggest that NRs TcHR51, TcDsf, TcHR78, TcHNF4 effect reproduction and egg maturation. In contrast, TcTll effects embryonic development.

3.2. Knock-down of NR mRNA levels in DsRNA injected larvae

As explained above, injection of DsRNA for most NRs showed recognizable effects on the development of *T. castaneum*. To determine whether DsRNA is effective in knocking down the levels of NR mRNA especially for those that did not cause visible phenotype, we monitored the levels of mRNA in larvae injected with TcE78,

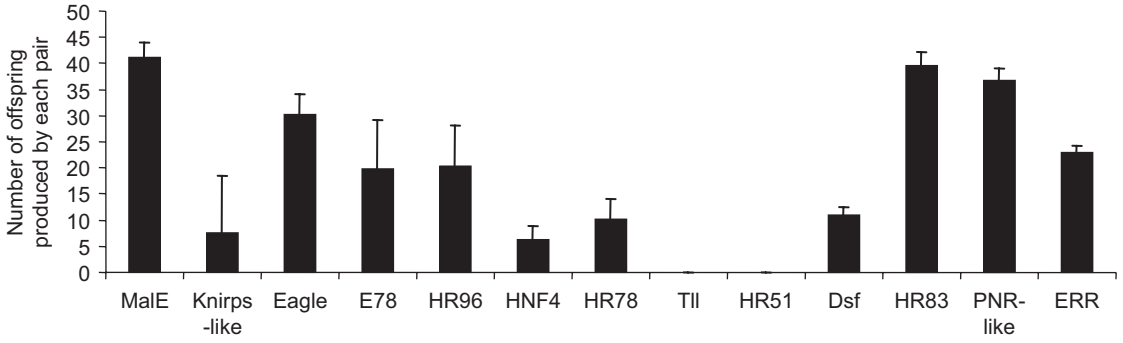

Fig. 2. The effect of knock-down of expression of NRs on the production of offspring. The healthy females developed from the larvae injected with NR DsRNA were mated with males developed from uninjected larvae over a 2-week period. The number of larvae present in the flour was counted at 1 week after removal of adults. Mean ± S.E. ($n = 4$) are shown.

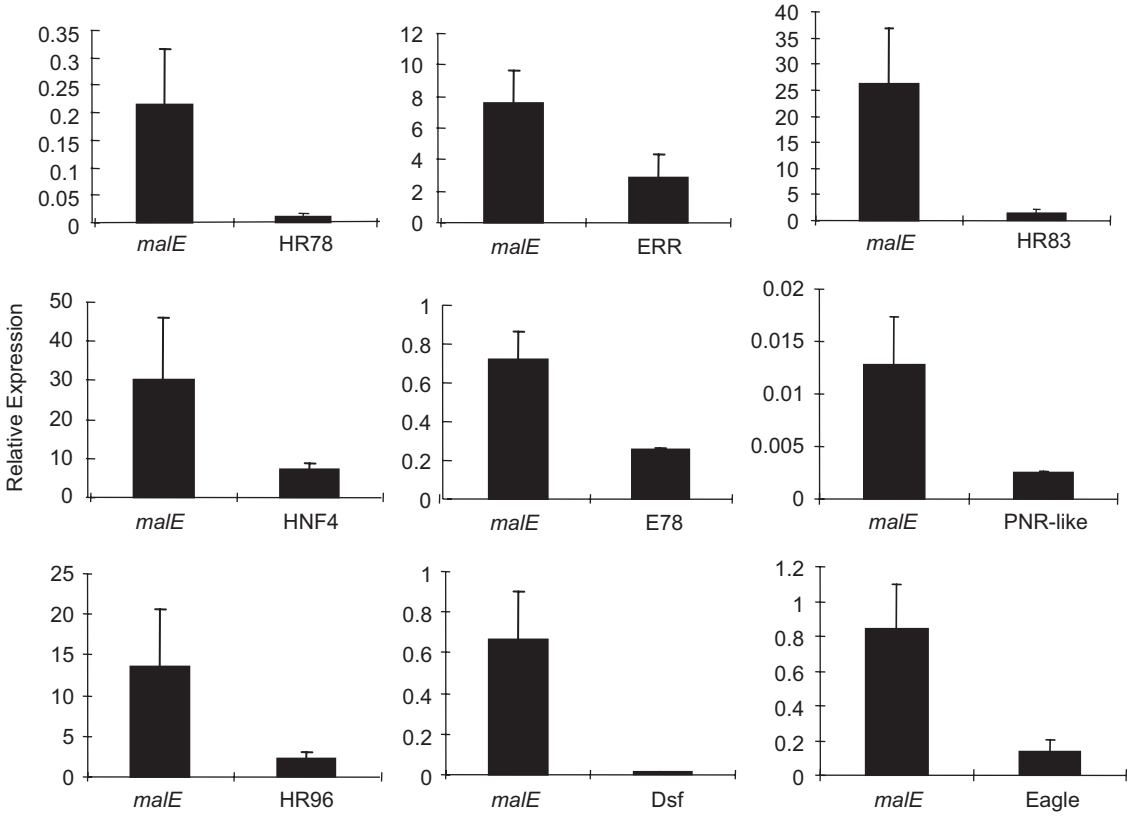

Fig. 3. Nuclear receptor or *malE* DsRNA was injected into 1-day-old final instar larvae. qRT-PCR was used to quantify mRNA levels at 72 h after DsRNA injection in larvae injected with nuclear receptor or *malE* DsRNA. Mean ± S.D. ($n = 3$) are shown.

TcHR96, TcHNF4, TcHR78,TcHR83, TcDsf, TcPNR-like, TcERR and TcEagle. The mRNA levels for all the NRs tested decreased in DsRNA injected larvae when compared to the levels in *malE* DsRNA injected larvae (Fig. 3). The TcPNR-like and TcEagle DsRNA prepared in the first round were not successful in knocking-down the expression of these genes. However, after changing the primers that amplified a different region of NR we were able to knock-down the expression of these two NRs also (Fig. 3). These new DsRNAS also did not affect metamorphosis or production of offspring. These data demonstrate that the mRNA levels of all NRs tested (including those that did not show obvious phenotype) were reduced in DsRNA injected larvae when compared to their levels in control larvae injected with bacterial *malE* DsRNA suggesting that the RNAi worked well but the reduction in expression levels of some of the NRs (Eagle, TcE78, TcHR83, TcHR96, TcPNR and TcERR) did not affect metamorphosis or production of offspring.

4. Discussion

The first major contribution of this study is the identification of 19 canonical and 2 Knirps family NRs in

the *T. castaneum* genome. Overall, the NRs are well conserved in the four insects whose genomes have been sequenced. Eighteen of the 19 NRs identified in *T. castaneum* have homologues in *D. melanogaster*, *A. mellifera* and *An. gambiae* genomes. PNR-like NR has been identified in *A. mellifera* and the homologue for this NR is present in *T. castaneum* genome but not in *D. melanogaster* or *An. gambiae* genomes. Both *D. melanogaster* and *A. mellifera* genomes contain three Knirps family members. In *T. castaneum*, only two members of Knirps family could be identified. Only two Knirps family members were identified in *An. gambiae* genome as well. Comparison of amino acid identity between *T. castaneum* and *D. melanogaster* as well as between *T. castaneum* and human NRs in the DBD and LBD revealed some interesting characteristics of *T. castaneum* NRs (Table 3). The DBDs of all *T. castaneum* NRs showed higher amino acid similarity with the DBDs of *D. melanogaster* homologues when compared to the DBDs human homologues. Most of the LBDs of *T. castaneum* NRs except USP and HR78 showed higher amino acid similarity with the LBDs of *D. melanogaster* homologues when compared to the LBDs of human homologues. The LBD of USP identified in *T. castaneum* showed higher amino acid similarity with the LBD of human RXR (62%) compared to USP LBD from *D. melanogaster* (44%), *Ae. aegypti* (42%), *Choristoneura fumiferana* (41%), *Manduca sexta* (41%), *Bombyx mori* (39%) or *Chironomus tetans* (40%). The LBD of HR78 homologue identified in *T. castaneum* also showed higher amino acid similarity with human TR2 (45%) when compared to the LBD of DHR78 (32%). The LBDs of some of the NRs such as TcE78, TcHR3, TcEcR, TcDHR96, TcDsf, TcSVP, TcFTZ-F1, TcHR39 and TcHR4 showed much higher amino acid similarity with the LBDs of *D. melanogaster* and other insect homologues when compared to the LBDs of human and other vertebrate homologues. Therefore, these NRs could be good candidates for development of insecticides, because the ligands recognized by these insect receptors are likely different from the ligands recognized by their human homologues.

The second major contribution of this study is the determination of the function of some of the NRs identified in the genome of *T. castaneum*. Our RNAi data clearly showed that seven NRs, TcE75, TcHR3, TcEcR, TcUSP, TcFTZ-F1, TcHR4 and TcHR51 are critical for larval–pupal metamorphosis. Additional three NRs TcHR38, TcHR39 and TcSVP are important for both larval–pupal and pupal–adult metamorphosis. EcR–USP heterodimer of NRs mediates 20E action (Palli et al., 2005). Interestingly, many direct targets of the 20E–EcR–USP complex are members of the NR superfamily (King-Jones et al., 2005). Six NRs, *Drosophila* hormone receptor 3 (DHR3), *Drosophila* hormone receptor 4 (DHR4), *Drosophila* hormone receptor 39 (DHR39), E75, E78 and FTZ-F1 were shown to play key roles in 20E action (King-Jones and Thummel, 2005). EcR–USP heterodimer binds to ecdysteroids and initiate molting and metamorphosis. Four insecticides that bind to EcR–USP complex with high affinity and disrupt normal molting process are being used to control pests all over the world.

E75 is an early gene induced directly by 20E (Segraves and Hogness, 1990). In E75-null mutants that do not express all three isoforms arrest their development during oogenesis suggesting a role for E75 in female reproduction (Buszczak et al., 1999). E75A-specific null mutation results in lower ecdysteroid titers leading to a block in larval development (Bialecki et al., 2002). Recent studies have shown the presence of heme in the ligand-binding pocket of E75 and the oxidation state of this molecule controls E75 activity (Reinking et al., 2005). In addition, gases NO and CO can activate E75 after binding to heme (Reinking et al., 2005), this raises an interesting possibility of targeting E75 with gaseous substances for controlling stored grain pests.

DHR3 and DHR4 are delayed-early genes that require some 20E induced protein synthesis for their maximal levels of expression (Koelle et al., 1992; King-Jones et al., 2005). DHR3 and DHR4 act as repressors of early ecdysone-induced genes and inducers of the βFTZ-F1, a mid-prepupal competence factor (Yamada et al., 2000). βFTZ-F1 is necessary for stage-specific response of 20E. Mutation analyses showed that ftz-f1 is essential for viability but DHR39 a closely related receptor is not essential; the null mutants are viable and fertile (Horner and Thummel, 1997; Yamada et al., 2000). Except for E78, all the other five NRs shown to be involved in 20E action play critical roles in metamorphosis of *T. castaneum*.

The RNAi studies in *T. castaneum* reported here clearly demonstrated the importance of SVP, HR38 and HR39 in metamorphosis. These three NRs are important for both larval–pupal as well as pupal–adult metamorphosis. The *D. melanogaster* SVP is important for development of photoreceptor cells of ommatidium, neuronal tissues, fat body, Malpighian tubules and circulatory system (Mlodzik et al., 1990; Hoshizaki et al., 1994; Broadus and Doe, 1995; Qiu et al., 1997; Kerber et al., 1998; Lo and Frasch, 2001). The SVP heterodimerizes with USP and can compete for EcR:USP DNA binding (Zelhof et al., 1995). The DHR38 mutants in *D. melanogaster* also die at the end of metamorphosis and DHR38 is required for adult cuticle formation (Kozlova et al., 1998). DHR38 is expressed throughout development and can heterodimerize with USP (Fisk and Thummel, 1995; Sutherland et al., 1995). The HR39 is related to fly FTZ-F1 and human liver receptor homologue 1 (LRH1) and steroidogenic factor 1. In contrast to RNAi data in *T. castaneum* where TcHR39 knock-down caused mortality during both larval–pupal as well as pupal–adult metamorphosis, the DHR39 mutants are viable and fertile in the fruit fly, *D. melanogaster* (Horner and Thummel, 1997).

Based on the function of their homologues in humans and nematodes, NRs, tailless (Tll), dissatisfaction (Dsf), HR51 and HR83 likely to function in development and embryogenesis. The tll, a gap gene plays a key role in the

establishment of sub-domains of embryo (Steingrimsson et al., 1991). The function of Tll during embryogenesis has been conserved in *T. castaneum* also because no first instar larvae hatched from the eggs laid by the females developed from larvae injected with TcTll DsRNA. The dsf is expressed in both sexes in a subset of neurons and mutations in this gene affect adult sexual behavior (Mlodzik et al., 1990; Finley et al., 1998). The Dsf appeared to be important for reproduction and egg maturation in *T. castaneum* because the females developed from larvae injected with TcDsf DsRNA laid fewer eggs when compared to the number of eggs laid by females developed from larvae injected with *malE* DsRNA.

In this study, we used RNAi and showed that 14 out of 19 canonical NRs and non-canonical NR Knirps-like play key roles in metamorphosis, reproduction and embryogenesis. The Knirps family member, Eagle, and five canonical NRs, TcE78, TcHR83, TcHR96, TcPNR-like and TcERR did not show detectable effect on metamorphosis or production of offspring. DHR96 was shown to be activated by CAR (constitutive androstane receptor) agonist CITCO (Palanker et al., 2006). *D. melanogaster* DHR96 null mutant displayed increased sensitivity to the sedative effects of Phenobarbital and DDT. The mutation also caused defects in the expression of phenobarbital-regulated genes suggesting a role for DHR96 in xenobiotic response (King-Jones et al., 2006). The PNR-like NR present in *T. castaneum* and *A. mellifera* is closely related to DHR51 and DHR83 and these three NRs in turn are related to human photoreceptor-specific nuclear receptor (PNR). It is interesting that only TcHR51 but not TcPNR-like or TcHR83 RNAi showed effects on larval–pupal metamorphosis as well as egg development. Recent studies in *D. melanogaster* demonstrated widespread transient activation of GAL4-ERR in the mid-third instar as the larvae prepare for entry into metamorphosis suggesting that ERR may play a role in metamorphosis (Palanker et al., 2006). In our RNAi studies, the TcERR knock-down did not affect metamorphosis, reproduction or embryonic development in *T. castaneum*. Further studies are required to determine the function of TcHR96, TcHR83, TcPNR-like and TcERR. These initial identification and characterization of *T. castaneum* NRs lay a solid foundation for the future detailed analysis on these important signal transduction molecules.

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There is no conflict of interest.

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